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Glucose transport by uterine plasma membranes

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Uterine plasma membrane preparations were obtained by centrifugation on discontinuous sucrose gradients. The specific activity of the plasma membrane marker 5′-nucleotidase was increased 10-fold while the specific activity of glucose-6-phosphatase was increased 3-fold. Electron microscopy showed mainly closed vesicles having diameters mainly in the range of 0.1 to 0.4 μm and an absence of other recognizable organelles such as mitochondria. D-Glucose transport was inhibited by sulfhydryl reagents, phloretin, and cytochalasin B. Uptake was prevented at high osmotic pressures. The K_m of glucose transport was 12.2 ± 1.1 mM. Studies of the inhibition of [^{3}H]cytochalasin B binding by D-glucose indicated that the value of the K_d of the cytochalasin B-transporter complex was larger than 1 μM. These data demonstrate the potential usefulness of these preparations in the study of glucose transport in rat uterus and its control by steroid hormones.

Introduction

Some of the effects of estradiol on the uterus of ovariectomized rats include increased amino acid [1–4], glucose [5–7] and RNA precursor [8] uptake as well as increased vascular permeability [9]. Uterine cell surface glycoproteins are altered as determined by changes in lectin binding [10,11]. All of these effects were observed to occur within 4 h after exposure to estradiol. These phenomena appear to involve actions at the plasma membrane.

Of particular interest to the authors is the stimulation in glucose transport. Whether this effect occurs through an increase in the intrinsic activity of the existing transport protein or through changes in the amount of transport protein in the plasma membrane is not known. It has been demonstrated that cycloheximide, an inhibitor of protein synthesis, can block the stimulation in glucose transport by estradiol, as measured by the accumulation of 2-deoxyglucose 6-phosphate in uterus in organ culture [7]. There is increased protein synthetic activity during this early time period although the synthesis of significant quantities of protein does not begin until about 4–6 h after estradiol exposure [12,13]. In order to investigate the mechanism of the regulation of glucose transport in uterus by steroid hormones, characterization of glucose transport in uterine tissue is an essential first step.

In this paper we describe certain properties of the glucose transport system in uterine plasma membranes. The presence of a glucose transport protein in the plasma membrane vesicles was indicated by stereospecific uptake of D-glucose and its inhibition by classic glucose transport inhibitors, e.g., cytochalasin B, phloretin, and sulfhydryl reagents. In addition, glucose transport into uterus, as measured by phosphorylation of 2-deoxyglucose, also was inhibited by cytochalasin B but not by cytochalasin E.

Methods

Materials. L-[1-^{14}C]Glucose (47 mCi/mmol), [4-^{3}H]cytochalasin B (10.3 Ci/mmol), and 2-deoxy-D-[G-^{3}H]glucose (8.3 Ci/mmol) were purchased from New England Nuclear, Boston, MA. D-[U-^{14}C]Mannitol (210 mCi/mmol), D-[1-^{14}C]glucose 6-phosphate (225 mCi/mmol) and D-[1-^{3}H]glucose (25 Ci/mmol) were purchased from ICN Pharmaceuticals, Inc., Irvine, CA. Nonradioactive cytochalasins B and E, phloretin, and *N*-ethylmaleimide were purchased from Sigma Chemical Co., St. Louis, MO. Sucrose was obtained from Schwarz/Mann, Inc., Spring Valley, N.Y. Liquid scintillation counting solution, BetaPhase, was obtained from WestChem, Inc., San Diego, CA. Other reagents were obtained from usual sources at the highest purity available.

Buffers. Buffer 1 consisted of 0.01 M Tris-HCl (pH 8.1), 0.1 mM EDTA and 4 mM sodium azide. Buffer 2 was Buffer 1 which also contained 0.25 M sucrose. Buffer 3 was Buffer 1 which also contained 1 mM magnesium chloride and 1 mM calcium chloride. Stop Solution was Buffer 3 which also contained 1 mM mercuric chloride.

Transport assays. Stock ethanol solutions of D-[^{3}H]glucose and L-[^{14}C]glucose were blown to dryness in a stream of nitrogen and then resuspended in Buffer 3 containing 0.1 mM D- and L-glucose each unless otherwise indicated, specific activity 4.65 dpm/fmol and 2.37 dpm/fmol, respectively. Five microliters of this solution were incubated with 10 to 15 μl of the membrane suspension (approx. 50 μg protein/assay) in a total volume of 25 μl for an appropriate time period at 20°C. Uptake was terminated by the addition of 3 ml of ice-cold Stop Solution. The mixtures were filtered through 0.65 μm Millipore filters (DAWP) and washed three times with 3 ml of ice-cold Stop Solution. The moist filters were dissolved in 1.0 ml methyl cellosolve during 10 min at room temperature. To this solution was added 14 ml of BetaPhase scintillation solution for the determination of radioactivity. Uptake is defined as the retention of any solute by membranes. D-Glucose transport is defined as D-glucose uptake minus L-glucose uptake. Stereospecific transport was only 20% of the total amount of sugar taken up. D-[^{14}C]Mannitol could be used in place of L-glucose with identical results. Cold stop solution stopped both specific uptake and efflux. D-Glucose transport rates were measured during a 7-s incubation.

Glucose transport in intact uterus was estimated by measuring the amount of 2-deoxyglucose 6-phosphate formed during a 30 min incubation of uterine horn with 2 ml of 0.1 mM D-2-[^{3}H]deoxyglucose, specific activity 1.76 dpm/pmol, in 0.15 M NaCl and 0.02 M potassium phosphate buffer at pH 7.0. Cytochalasin B was added to certain incubations as indicated. Horns were removed, rinsed twice with the incubation buffer without 2-deoxyglucose and homogenized with 2 ml of 5% trichloroacetic acid with a Polytron homogenizer. Approx. 3000 dpm of D-[^{14}C]glucose 6-phosphate were added just before homogenization as a recovery indicator. The mixture was centrifuged at 15000 $\times g$ for 15 min. The supernatant solution was extracted three times with ethyl ether (to remove trichloroacetic acid). The aqueous layer was then passed through a 0.7 $\times$ 1.2 cm column containing AG 1-X2, 200–400 mesh (BioRad), which had been prepared by washing with several ml of 1 M HCl, followed by several ml of water. The sample was followed by three 1-ml aliquots of water. Radioactive 2-deoxyglucose 6-phosphate was eluted with two 1-ml aliquots of 1 M HCl. The eluates were combined and added to 15 ml of BetaPhase scintillation fluid. Tritium dpm values were corrected to 100% recovery of D-[^{14}C]glucose 6-phosphate.

Membrane preparation. All procedures were performed at 0–5°C. Uterine membranes were prepared from intact Sprague-Dawley-derived rats (160–180 g) obtained from Small Animal Supply Co., Omaha, NE. The uteri (4–16) were excised and placed in ice-cold Buffer 2. The uteri from animals in random stages of the estrous cycle were stripped of fat, cut into small pieces with a razor blade and homogenized, two uteri at a time, in a 10 ml glass-teflon homogenizer using 20–25 strokes. The pestle was polished with emery cloth to increase the clearance with the glass from approx. 0.15 mm to 0.25 mm. Homogenization with a Polytron homogenizer equipped with a PT-10 generator at full speed twice for 10 s each gave results comparable to the glass teflon homogenizer. The homogenate was centrifuged at 2000 $\times g$ for 5 min. (Centrifugal forces were estimated

at the bottom of the tube.) The supernatant solution was saved and the pellet was washed by resuspending it in 4 ml of the same buffer and centrifuging again at $2000 \times g$ for 5 min. The supernatant solutions were combined and centrifuged at $210000 \times g$ for 45 min. The pellet was resuspended in 2 ml of Buffer 2 and applied to the surface of a discontinuous gradient consisting of 2 ml of 1.1 M sucrose in Buffer 1 and 0.5 M sucrose in the same buffer. After centrifugation at 41000 rpm ($286000 \times g$) in an SW-41 swinging bucket rotor for 90 min, the turbid material at the interface was removed with a pipet, diluted with an equal volume of Buffer 1, and centrifuged at $210000 \times g$ for 45 min. The pellet was resuspended in Buffer 3 to give a protein concentration of approx. 3 mg/ml.

Enzyme determinations. The enzymes 5′-nucleotidase and glucose-6-phosphatase were measured as described by Müller et al. [20] by measuring the rate of release of phosphate from 5′-AMP and glucose 6-phosphate [14]; ouabain-sensitive sodium/potassium ATPase was measured by the method of Bers [15]; phosphodiesterase was measured by the method of Kelley et al. [16]; and cytochrome *c* oxidase was measured by the method of Stocco and Hutson [17]. Protein was determined according to Bradford [18] using a commercially available protein assay reagent (obtained from BioRad Laboratories, Richmond, CA) with Fraction V bovine albumin (Sigma Chemical, Co.) used as the standard.

Electron microscopy. Membrane pellets were fixed with 2.5% glutaraldehyde in 0.5 M potassium phosphate buffer (pH 7.3) for a minimum of 2 h. The pellets were then post-fixed for 1 h in 2% osmium tetroxide (pH 7.0), dehydrated through a graded series of ethanol solutions, and embedded in an epon epoxy resin. Sections were cut on a Sorvall MT-2B ultramicrotome, stained in uranyl acetate and lead citrate, and examined in a Hitachi H-600 electron microscope at 75 kV.

Curve fitting. Analysis of kinetics experiments was accomplished by an iterative nonlinear curve-fitting procedure performed on-site with an IBM 370 computer using a program originating from SAS Institute, Box 8000, Cary, N.C., 27511. The results are expressed as estimates of the kinetic parameters ± the asymptotic standard errors.

Results and Discussion

Uterine plasma membranes were prepared by centrifugation on a discontinuous sucrose gradient consisting of 0.5 M sucrose layered over 1.1 M sucrose. Plasma membrane markers were located mainly at the interface between the two solutions. A typical purification summary is shown in Table I. This procedure is similar to the procedures of Kidwai et al. [19] and Müller et al. [20]. Enzyme markers determined in several preparations were: 5′-nucleotidase, ouabain-sensitive ATPase, and phosphodiesterase (plasma membrane); glucose-6-phosphatase (endoplasmic reticulum); and cytochrome *c* oxidase (mitochondria). The ATPase and 5′-nucleotidase marker enzymes were purified about 10-fold while phosphodiesterase was purified only about 3-fold. The increase in specific activity of cytochrome *c* oxidase indicated some contamination with mitochondria.

Electron micrographs (Fig. 1) of these preparations showed mainly closed vesicles of varying size apparently from plasma membranes. Other recognizable structures such as mitochondria or contractile elements were not observed. In other photographs, it could be seen that the pelleted membranes were homogeneous from the top to the bottom of the pellet. The intravesicular volume of 1.8 μl/mg protein was determined from uptake which was allowed to proceed for 90 min.

These preparations were capable of stereo-

TABLE I

SPECIFIC ACTIVITIES OF MEMBRANE MARKER ENZYMES

Specific activities are expressed as micromoles of product formed/h per mg protein except for cytochrome *c* oxidase, which is expressed as the change in absorbance at 550 nm/min per mg protein. Values are expressed ± S.D. (number of preparations). Membranes were collected at the interface between 0.5 M sucrose and 1.1 M sucrose.

Enzyme	Preparation	
	Homogenate	Membranes
5′-Nucleotidase	5.3 ±0.6 (6)	49 ±11 (6)
Phosphodiesterase	0.32 ± 0.08 (2)	1.0 ± 0.3 (2)
Ouabain-sensitive ATPase	6.24 (1)	57.8 (1)
Glucose 6-phosphatase	1.6 ±0.8 (2)	5.7 ± 1.7 (2)
Cytochrome *c* oxidase	0.49 ± 0.17 (2)	0.81 ± 0.28 (2)
Protein, mg/uterus	15.6 ±2.0 (6)	0.39 ± 0.15 (6)

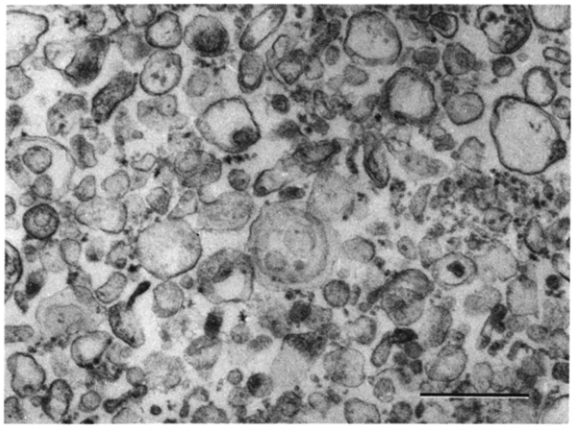

Fig. 1. Electron micrograph of a thin section prepared from the pellet of membranes isolated as described in Methods. The gradient consisted of a 2-ml layer of 0.6 M sucrose over 2 ml of 1.1 M sucrose. The bar represents 500 nm.

specific transport, as expected for a carrier-mediated process. Shown in Fig. 2 is a typical time-course of the transport of D[^{3}H]glucose. It is assumed that L-glucose uptake represents nonspecific uptake due to leakiness of the vesicular membrane while D-glucose uptake represents both nonspecific uptake and specific uptake mediated by a transport protein. Therefore the difference between the two curves (Fig. 2) represents stereospecific, carrier-mediated, transport of D-glucose. By the end of a 90-min incubation complete equilibration was reached. The half-time of D-glucose transport was 20 ± 4 s. Membrane vesicles prepared from ovariectomized rats had similar transport properties.

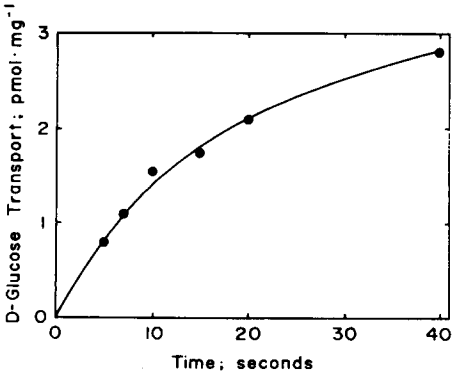

Fig. 2. Time-course of glucose transport by uterine membranes. Glucose transport was measured as described in Methods.

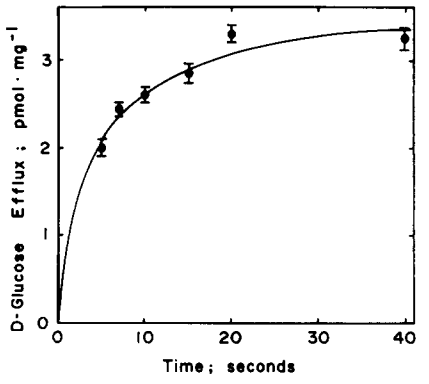

Fig. 3. Efflux of D-glucose from previously-loaded membrane vesicles. Reaction mixtures identical to those described for the measurement of glucose uptake were allowed to incubate at 20°C for 90 min, allowing D- and L-glucose to equilibrate in the vesicles. Three ml of Buffer 3 was added and the incubation was continued for the indicated time and filtered and washed three times with cold Stop Solution. Radioactivity was determined as described. D-Glucose efflux is defined as pmol L-glucose retained by the filter minus pmol D-glucose retained by the filter. Values are positive indicating that efflux of D-glucose was more rapid than the efflux of L-glucose.

These preparations were also capable of transporting glucose stereospecifically in both directions. Vesicles were loaded with both D- and L-glucose during a 90-min incubation and then diluted with buffer and filtered at various times after dilution. It can be seen in Fig. 3 that the loss of D-glucose was more rapid than the loss of L-glucose having a half-time of 4–8 s. This rapid loss of D-glucose is consistent with carrier-mediated transport system which functions in both directions.

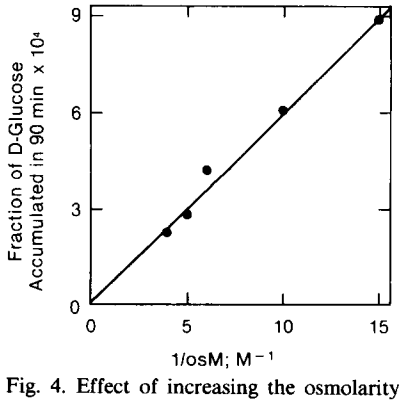

Fig. 4. Effect of increasing the osmolarity of glucose uptake. Medium osmolarity was adjusted with D-mannitol in Buffer 3. The line was fitted by linear regression and had $r > 0.99$.

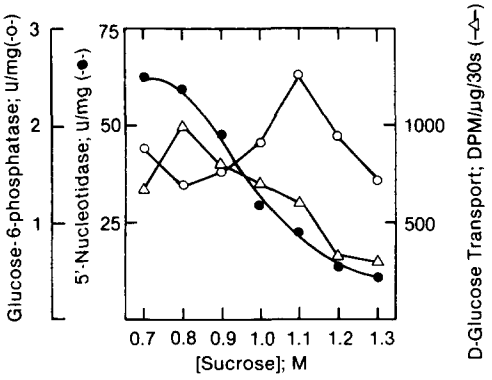

Fig. 5. Fractionation of uterine membranes on a discontinuous sucrose gradient. Crude membranes were layered over a sucrose gradient. Crude membranes were layered over a sucrose gradient. Crude membranes were layered over a sucrose gradient and centrifuged at 41000 rpm (286000 × g) in an SW-41 swinging bucket rotor for 1.5 h. The gradient consisted of layers of 1.3 ml of each of nine solutions in Buffer 1 which had sucrose concentrations ranging from 0.5 M to 1.3 M in increments of 0.1 M. In some experiments, no membranes were obtained at the interface between 0.5 M and 0.6 M. Turbid material at each interface was collected, diluted with Buffer 1 and centrifuged at 210000 × g for 45 min. Pellets were resuspended in Buffer 3 and assayed as indicated. Specific activities of 5'-nucleotidase (●———●), glucose 6-phosphatase (○———○) and D-glucose transport (△———△) were measured.

Membranes prepared by these procedures behaved according to the van't Hoff relationship in that the uptake of D-glucose was markedly reduced by increasing the osmolarity of the medium. The line shown in Fig. 4 extrapolates to zero indicating that there was no detectable binding of glucose to the membranes.

Shown in Fig. 5 is the distribution of membrane markers on a discontinuous sucrose gradient which was prepared from a series of sucrose solutions in Buffer 1 ranging from 0.5 M at the top of the centrifuge tube to 1.3 M at the bottom of the tube. It can be seen that there was significant D-glucose transport into membranes associated with the same regions of the gradient in which the plasma membrane marker, 5'-nucleotidase, was found with little D-glucose transport into membranes associated with the endoplasmic reticulum marker. Thus for purposes of this study, discontinuous gradients were constructed which maximized yield of vesicles which had D-glucose uptake properties. By selecting slightly different sucrose solutions for the gradient, e.g., 0.6 M and 0.9 M, a 15-fold increase

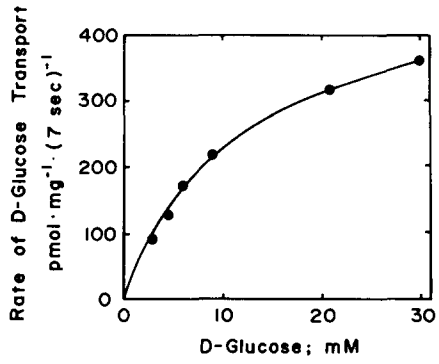

Fig. 6. D-Glucose transport as a function of D-glucose concentration. The rate of D-glucose transport was measured as described in Methods. A K_m value of 12.2 ± 1.1 mM was determined by computer fit as described.

in the specific activity of 5'-nucleotidase was obtained with only a 2-fold increase in the specific activity of glucose-6-phosphatase. With this gradient there was an overall 4-fold increase in the specific activity of phosphodiesterase and an overall slight decrease in the specific activity of cytochrome c oxidase (not shown). In this case there was an additional 40% decrease in the yield of plasma membranes. Longer centrifugation times did not improve the yield or purity of plasma membranes. Fifteen-fold purification is typical of such preparations [21].

D-Glucose uptake was found to be saturable having a K_m value of 12.2 ± 1.1 mM, as shown in fig. 6.

Stereospecific uptake was inhibited by several known inhibitors of glucose transport (Table II) including phloretin [22], cytochalasin B [23] and

TABLE II

INHIBITORS OF GLUCOSE TRANSPORT BY UTERINE PLASMA MEMBRANES

Inhibitors were added to the membranes 15 min before addition of radiolabel. The complete reaction mixture was incubated for 2 min prior to filtration.

Additions	Concn. (mM)	% Control
None	–	(100)
N-Ethylmaleimide	15	15
Phloretin	0.5	20
Cytochalasin B	0.005	21
Mercuric ion	1	25

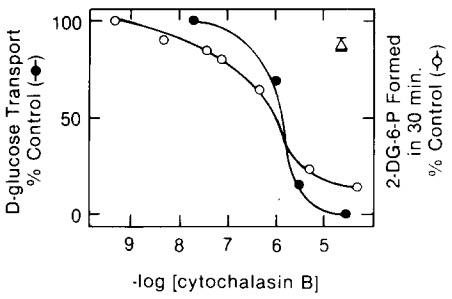

Fig. 7. Inhibition of D-glucose transport in membranes by cytochalasin B (●———●) and inhibition of 2-deoxyglucose 6-phosphate formation in whole uterine horn by cytochalasin B (○ ——— ○) and cytochalasin E (△). D-Glucose transport was measured after a 7 s incubation as described. The formation of 2-deoxyglucose 6-phosphate during a 30-min incubation of intact uterine horn is described in Methods.

by sulfhydryl reagents such as mercuric ion and *N*-ethylmaleimide [24]. The inhibitions are underestimated due to the long incubation time. These data indicate that the glucose transport system in uterus is similar to the systems isolated from muscle [25], adipocytes [26], erythrocytes [27], etc.

The inhibition by cytochalasin B suggested the possibility of measuring the amount of transport protein from [^{3}H]cytochalasin B binding studies. As seen in Fig. 7, the concentration of cytochalasin B required for 50% inhibition of D-glucose transport by plasma membrane vesicles and in intact uterus was 1–3 μM. This value is about 10-fold higher than that usually measured in plasma membranes from other tissues [28]. Measurements of the K_d of cytochalasin B binding which could be displaced by D-glucose was unsuccessful because of high amounts of nonspecifically bound cytochalasin B, even in the presence of cytochalsin E, which did not inhibit D-glucose transport. The data indicated that the value of the K_d was larger than 1 μM.

References

1 Noall, M.W. and Allen, W.M. (1961) J. Biol. Chem. 236, 2987–2990
2 Riggs, T.R., Pan, M.W. and Feng, H.W. (1968) Biochim. Biophys. Acta 150, 92–103
3 Roskoski, R. and Steiner, D.F. (1967) Biochim. Biophys. Acta 135, 727–731
4 Walters, M.R., Lawrence, A.L. and Hazelwood, R.L. (1975) Biol. Reprod. 12, 383–388
5 Walters, M.R., Lawrence, A.L. and Hazelwood, R.L. (1981) Endocrinology 108, 1915–1919
6 Roskoski, R. and Steiner, D.F. (1967) Biochim. Biophys. Acta 135, 717–726
7 Smith, D.E. and Gorski, J. (1968) J. Biol. Chem. 243, 4169–4174
8 Means, A.R. and Hamilton, T.H. (1966) Proc. Natl. Acad. Sci. U.S.A. 56, 686–693
9 Peterson, R.P. and Spaziani, E. (1971) Endocrinology 89, 1280–1286
10 Pietras, R.J. and Szego, C.M. (1975) Endocrinology 97, 1445–1454
11 Murphy, C.R. and Rogers, A.W. (1981) Cell Biophys. 3, 305–320
12 O'Malley, B.W. and Means, A.W. (1974) Science 183, 610–620
13 Noteboom, W. and Gorski, J. (1963) Proc. Natl. Acad. Sci. 50, 250–255
14 Aronson, N.N. and Touster, O. (1974) Methods Enzymol. 31, 90–102
15 Bers, D.M. (1979) Biochim. Biophys. Acta 555, 131–146
16 Kelley, S.J., Dardinger, D.E. and Butler, L.G. (1975) Biochemistry 14, 4983–4988
17 Stocco, D.M. and Hutson, J.C. (1980) Cancer Res. 40, 1486–1492
18 Bradford, M. (1976) Anal. Biochem. 72, 248–254
19 Kidwai, A.M., Radcliffe, M.A. and Daniel, E.E. (1971) Biochim. Biophys. Acta 233, 538–549
20 Müller, R.E., Johnston, T.C. and Wotiz, H.W. (1979) J. Biol. Chem. 254, 7895–7900
21 Neville, D.M., Jr. (1975) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 3, pp. 1–49, Plenum Press, New York
22 LeFevre, P.G. (1954) Symp. Soc. Exp. Biol. 8, 118–135
23 Lin, S. and Spudich, J.A. (1974) J. Biol. Chem. 249, 5778–5783
24 LeFevre, P.G. (1948) J. Gen. Physiol. 31, 505–527
25 Klip, A. and Walker, D. (1983) Arch. Biochem. Biophys. 221, 175–187
26 Illiano, G. and Cuatrecasas, P. (1971) J. Biol. Chem. 246, 2472–2479
27 Baldwin, J.M., Gorga, J.C. and Lienhard, G.E. (1981) J. Biol. Chem. 256, 3685–3689
28 Plagemann, P.G.W., Graff, J.C. and Wohlhueter, R.M. (1977) J. Biol. Chem. 252, 4191–4202